Supporting Information

THRUST: translesion synthesis-driven hierarchical regulation using template-activator construct for Cas12a activity

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1. Experimental Section

1.1 Materials and reagents

All oligonucleotides (as shown in Supplementary Table S1-S5) were synthesized by Shanghai Sangon Biotechnology Co., Ltd (Shanghai, China), LbaCas12a Nuclease (Cpf1) and Buffer II was purchased from Novoprotein Scientific Inc. (Shanghai, China). Uracil-DNA glycosylase (UDG), T7 RNA polymerase (T7 RNAP), ribonucleoside triphosphates (rNTPs), human alkyl adenine DNA glycosylase (hAAG), human 8-oxoguanine-DNA glycosylase (hOGG1), flap endonuclease 1 (FEN1), human apurinic/apyrimidinic endonuclease (APE1), T4 polynucleotide kinase (T4 PNK), terminal deoxynucleotidyl transferase (TdT), nicking endonuclease (Nt.BbvCI), Exonuclease I (Exo I) were purchased from NEB (Beijing, China). 1-(3,7-Dihydroxy-10H-phenoxazin-10-yl) ethanone (Amplex Red) was obtained from Shanghai Bide Pharmatech Ltd. (Shanghai, China). Potassium Chloride (KCl), hemin, streptavidin (SA) was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Bottom plate, absorbent pad, sample pad, PVC backing plate was obtained from Shanghai Jiening Biotech Co., Ltd. (Shanghai, China). Nitrate cellulose membrane was obtained from Sartorius Scientific Instruments Co., Ltd. (Beijing, China). All other chemicals were of analytical reagent grade.

1.2 Apparatus

Fluorescence measurements were performed on an F-7100 fluorescence spectrofluorometer (Hitachi, Japan). The agarose gel electrophoresis (Shanghai Tanon HE-120) was performed in 3.5% agarose gel with 0.5 × TBE buffer at 100 V and the agarose gel electrophoresis image was captured on a Gel Doc EZ Imager system (Bio-Rad). The test strip was made using an XYZ dispensing platform (Shanghai Jinbiao HM2035).

1.3 Inhibition analysis of UDG

UDG inhibition assay was performed by incubating various concentrations of UGI with a fixed concentration of 0.03 $U \cdot mL^{-1}$ UDG in 1 × UDG buffer at 37°C for 15 min in advance. Then, 2 nM TAC-dU2 was added and incubated at 37 ℃ for 1 h.

Next, 30 U T7 RNAP, 0.1 mM rNTPs, and T7 RNAP buffer were added to make the system volume 30 μL and incubated at 37°C for 1 h. After that, 30 μL of the reaction mixture containing 10 nM Cas12a, 0.4 μM reporter probe G4, and Buffer II was introduced and incubated at 37℃ for 0.5 h. Finally, 200 nM hemin, 30 μM AR and 1 mM H_2O_2 were added to 100 μL of the reaction solution containing HEPES buffer (pH 7.3) and reacted at room temperature for 5 min. The fluorescence intensity at 598 nm was measured. The relative activities (RAs) of UDG was calculated according to: $RA\%=(F_i-F_0)/(F_t-F_0)\times 100\%$, where F_0 was the fluorescence intensity without UDG, F_t was the fluorescence intensity when UDG was present, and F_i was the fluorescence intensity with both UDG and UGI.

1.4 Cell culture and preparation of cell extracts

The cancer cell line (HeLa, A549 cells) was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, U.S.A.) supplemented with 10% fetal bovine serum (FBS, Invitrogen, U.S.A.) and 1% penicillin-streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2. The number of cells was measured by Countstar cell counter. The nuclear extracts were prepared using the nuclear extract kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. The obtained supernatant was subjected to a UDG activity assay.

1.5 The construction of G4-based test strip

The test strip was comprised of sample pad, absorbent pad, nitrocellulose membrane (NC membrane), and PVC backing plate. Streptavidin (SA) and SA-bio-G4 were separately sprayed on the NC membrane at a density of 1 $\mu L\cdot mL^{-1}$ to act as the T and C line using the XYZ dispensing platform. After that, the test strip was prepared by orderly attaching the sample pad and absorbent pad on the NC membrane with an overlap of about 2 mm. The prepared strips were further cut into 4 mm and stored at 4℃.

2. Supplementary Figures

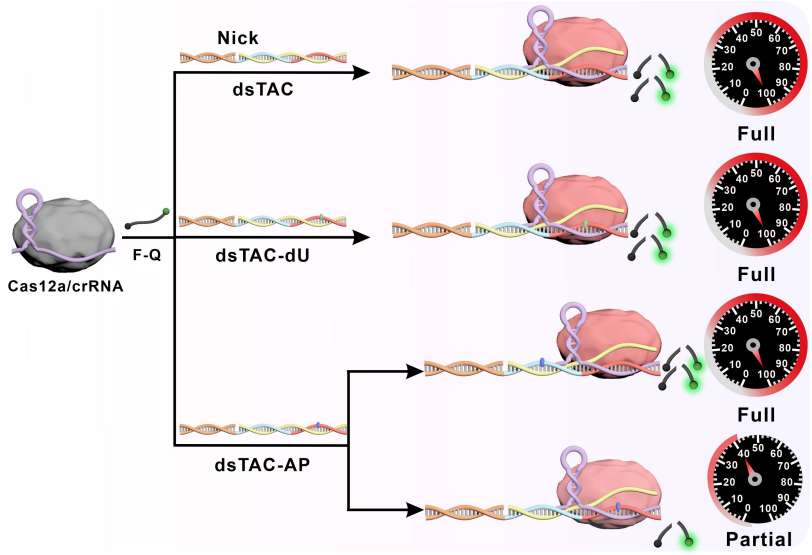

Figure S1. Schematic diagram of direct effects of Cas12a activation at the activator level in dsTAC. There was a nick structure between the T7 promoter and the complementary sequence of CRISPR array.

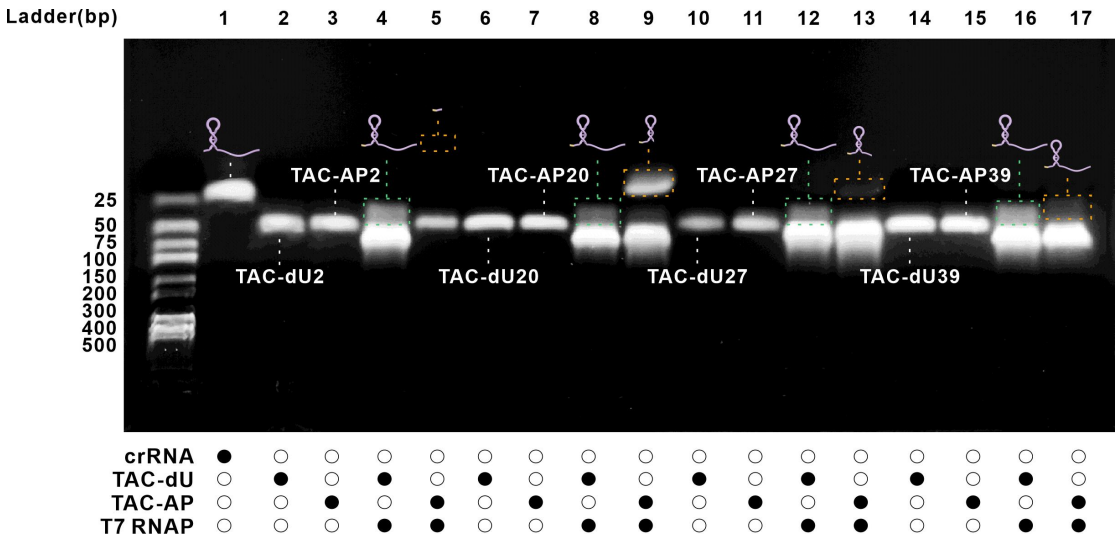

Figure S2. Agarose gel electrophoresis verified that the THRUST system transcribed different lengths of crRNA.

In Lane 4/ 8/ 12/ 16, TAC-dU2/TAC-dU20/TAC-dU27/TAC-dU39 acted as template, and T7 RNAP could transcribe across the dU lesion to obtain crRNA replicas. However, since the generated crRNA replicas were completely complementary to the template, it was difficult for the crRNA replica to dissociate from the template.[1] This tight binding caused the crRNA replica attached to the template to increase the number of TAC-dUs bases, thereby slowing down their migration rate in the gel. In addition, the crRNA replica must undergo a shedding process before electrophoretic migration, and this extra step also caused their migration rate to be slower than that of the crRNA control (Lane 1).[1,2] T7 RNAP loses its pairing signal when encountering the AP site, usually stops transcription before the site, and the transcription initiation mechanism of T7 RNAP, so the total length of the transcription product increases by 2 nt accordingly. In Lane 5, T7 RNAP was blocked at the AP2, resulting in interrupted transcription and the production of crRNA fragments of merely 4 nt, which made it difficult to observe this band in gel electrophoresis. In Lane 9, T7 RNAP was blocked at AP20 and 22 nt RNA fragments were generated. Since the fragments were short, the fragments were easier to dissociate from the template, so its band was brighter and clearer. In Lane 13, T7 RNAP was blocked at AP27, producing RNA fragments (29 nt) that were longer than 22 nt but shorter than crRNA replicas, thus the migration rate was slower than that in Lane 9 but faster than that in Lane 12.

Since the 29 nt fragments had stronger binding force with the template and took longer to dissociate, the brightness of the band was weaker than Lane 9. In Lane 17, the 41 nt crRNA fragments transcribed by TAC-AP39 were close to the crRNA replicas. Due to the increased number of base pairs, the binding force with the template was significantly enhanced, the RNA fragments obtained by dissociation were lowest and the migration speed was slower than Lane 13.

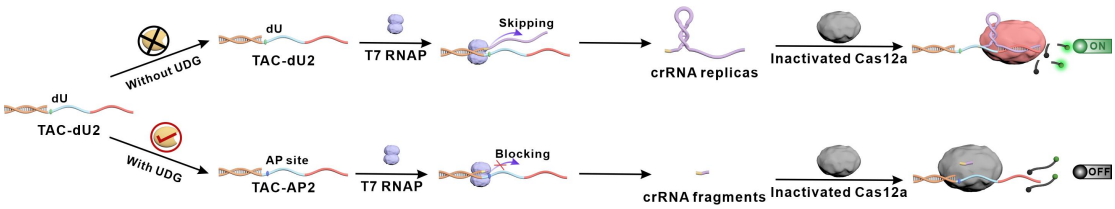

Figure S3. Schematic diagram of the "Dim down" signal output platform.

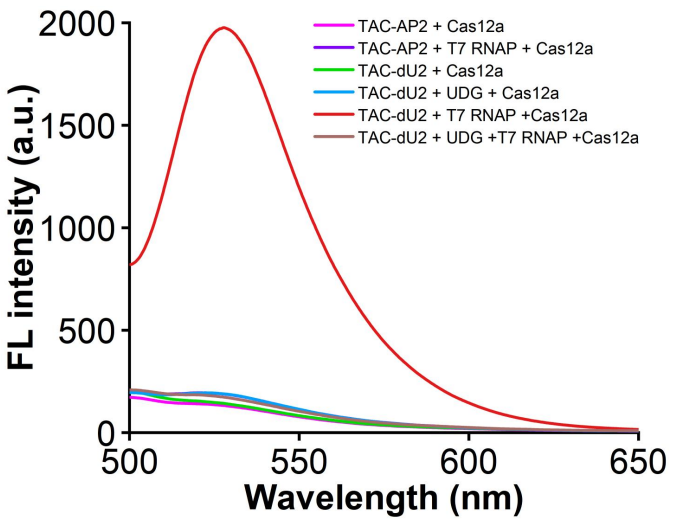

Figure S4. Proof-of-concept for THRUST system in "Dim down" platform. The pink and green curves represented the presence of only TAC-AP2 or TAC-dU2 in the system. In the absence of T7 RNAP, the transcription reaction could not proceed and no fluorescent signal was generated. The purple fluorescence curve indicated that when TAC-AP2 and T7 RNAP existed simultaneously, T7 RNAP could not bypass the AP site and only transcribe short crRNA fragments. As a result, the *trans*-cleavage activity of Cas12a could not be activated and no fluorescence was generated. The blue curve indicated that in the presence of UDG, but no transcription occurs in the absence of T7 RNAP, and therefore no activation of Cas12a. Conversely, the red curve demonstrated that under UDG free conditions, T7 RNAP could bypass dU and transcribe synthesis TAC-dU2 into a complete crRNA replica, thereby activating Cas12a to cleave the F-Q probe and generated a significant fluorescence signal. Like to the purple curve, the brown curve demonstrated that following the recognition and replacement of dU in TAC-dU2 with the AP site by UDG, T7 RNAP was only able to transcribe short crRNA fragments and was unable to activate Cas12a *trans*-cleavage activity, resulting in no fluorescent signal being generated.

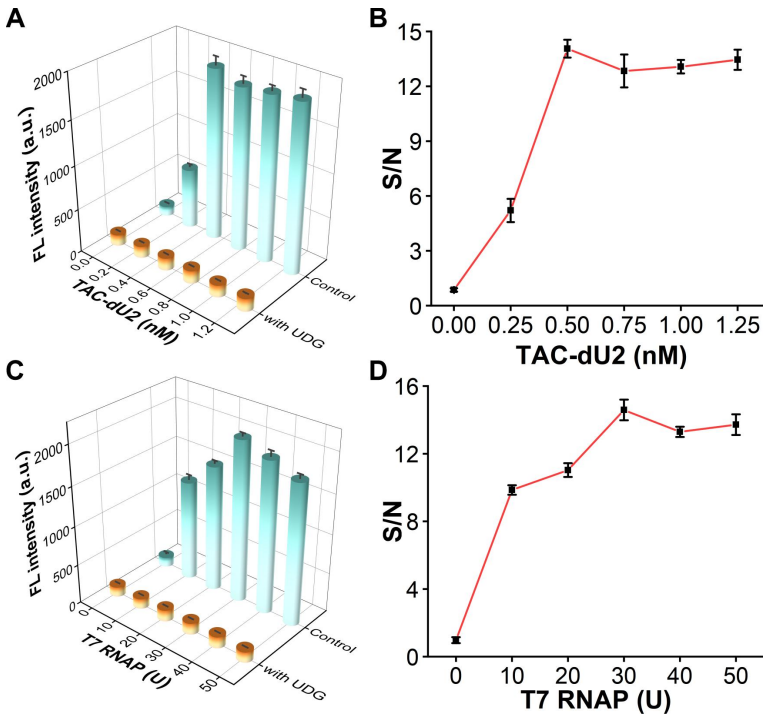

Figure S5. Optimization of THRUST system in " Dim down " platform. Optimization of (A) TAC-dU2 concentration and (B) the corresponding signal/noise ratio (S/N). Optimization of (C) T7 RNAP concentration and (D) the corresponding S/N.

The optimal conditions were: 0.5 nM of TAC-dU2, 30 U of T7 RNAP. These optimum conditions were adopted in further analysis.

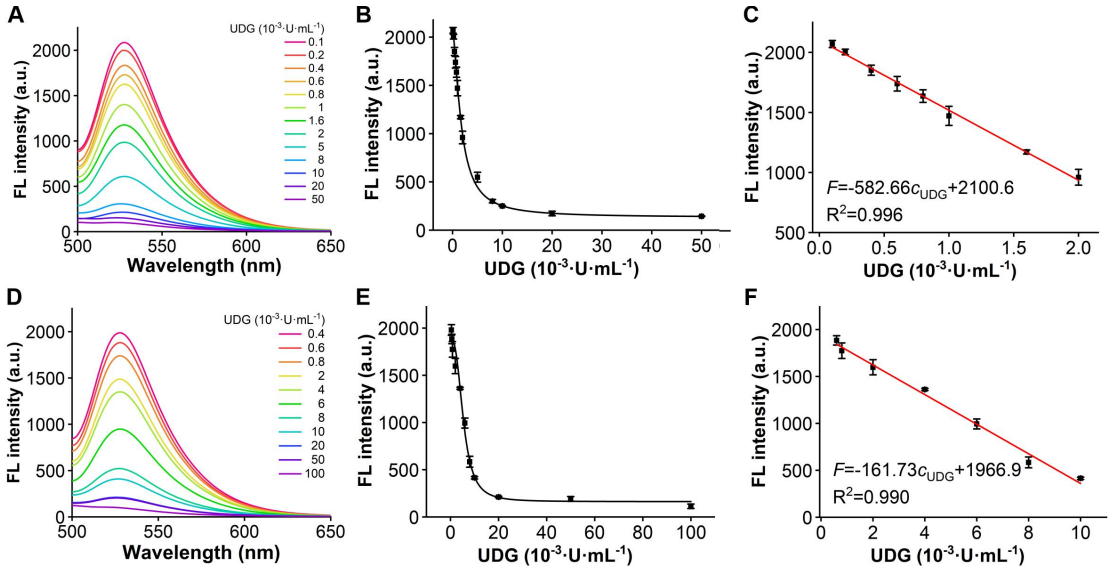

Figure S6. Quantification of THRUST system in "Dim down" platform. (A) Fluorescence emission spectra of THRUST system of the TAC-dU2 upon the addition of UDG. (B) Fluorescence intensity of UDG from 0.00010 to 0.050 U mL^{-1}. (C) The linear relationship between fluorescence intensity and UDG concentration ranging from 0.0001 to 0.0020 U mL^{-1}. (C) Fluorescence emission spectra of THRUST system of the dsTAC-dU2 upon the addition of UDG. (E) Fluorescence intensity of UDG from 0.0004 to 0.10 U mL^{-1}. (F) The linear relationship between fluorescence intensity and UDG concentration ranging from 0.0004 to 0.010 U mL^{-1}.

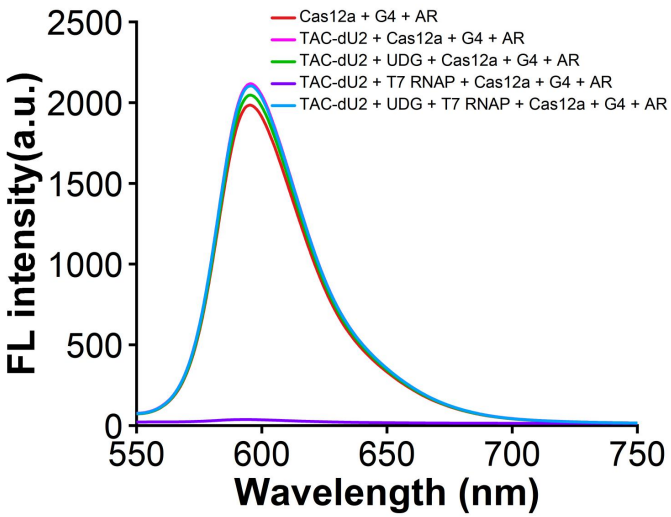

Figure S7. Proof-of-concept for THRUST system in "Light up" platform. In the absence of both TAC-dU2 and T7 RNAP (red curve), Cas12a was inactivated so that the complete G4 was not degraded. As a result, G4/hemin oxidized AR into resorufin, producing red fluorescence. When TAC-dU2 was present but T7 RNAP was not present (pink curve), no complete crRNA was synthesized, Cas12a was not activated and G4 remained intact, leading to the same red fluorescence. Similarly, when TAC-dU2 and UDG were present but without T7 RNAP (green curve), no complete crRNA was generated, resulting in red fluorescence. In contrast, when TAC-dU2 and T7 RNAP were present (purple curve), T7 RNAP could bypass the dU base to produce complete crRNA, activating Cas12a to cleavage G4, which prevented red fluorescence. When TAC-dU2, UDG and T7 RNAP were present (blue curve), UDG recognized dU and replaced it with AP site, blocking T7 RNAP and resulting in the synthesis of only short crRNA fragments. These fragments failed to activate Cas12a, allowing G4/hemin to oxidize AR into resorufin, producing red fluorescence.

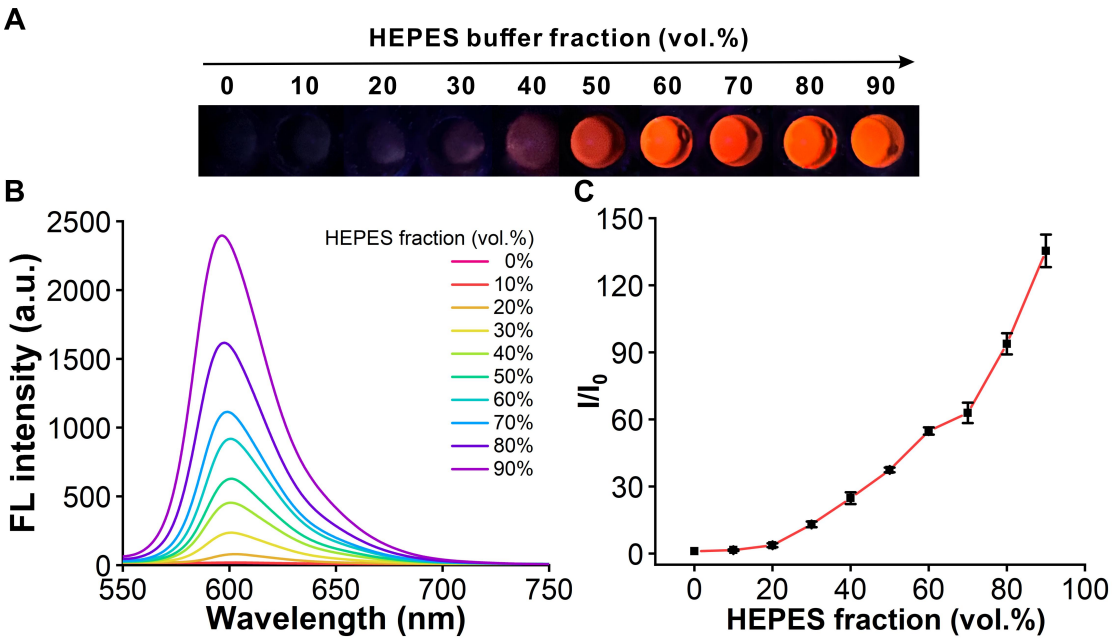

Figure S8. AIE curves for resorufin. (A) Photograph of resorufin in HEPES/DMSO mixtures with different fractions taken under 365 nm. (B) Fluorescence spectra of resorufin generated by G4/hemin-catalyzed oxidation of AR in HEPES/DMSO mixtures with different HEPES fractions. (C) Relative fluorescence intensity (I/I_0) of the resorcinol generated by the oxidation of AR by G4/hemin relative to the HEPES fractions.

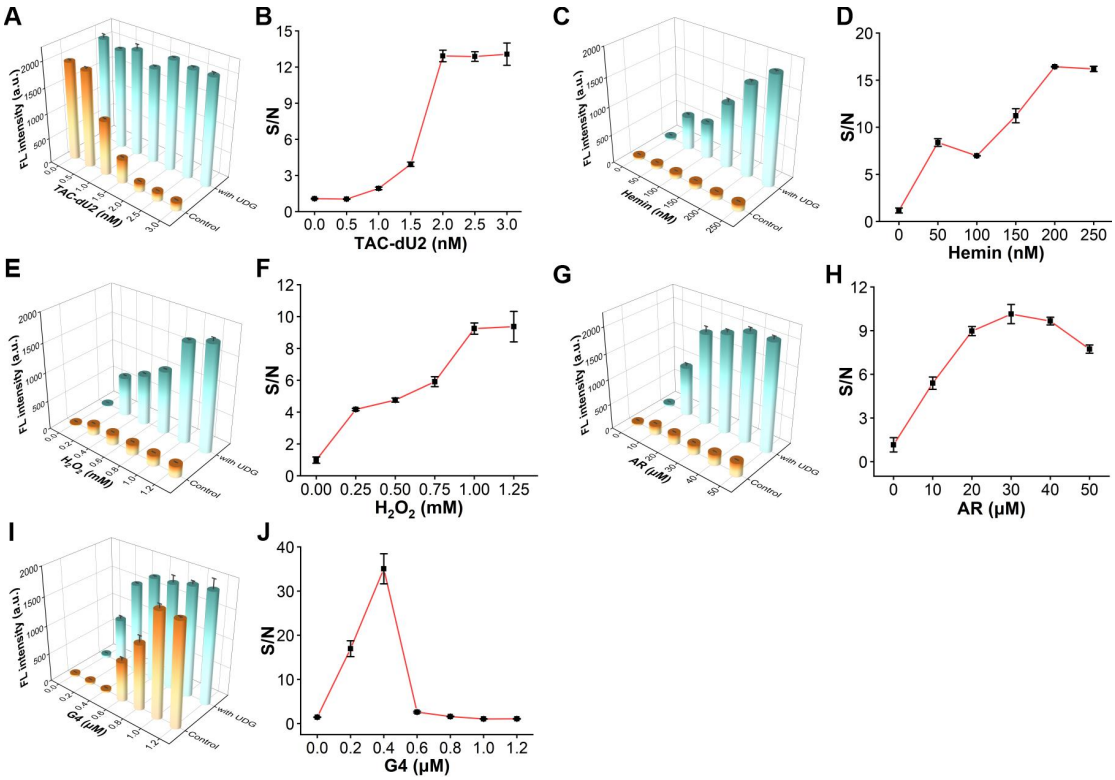

Figure S9. Optimization of THRUST system in "Light up" platform. The concentration of (A) TAC-dU2 and (B) the corresponding S/N. The concentration of (C) hemin and (D) the corresponding S/N. The concentration of (E) H_2O_2 and (F) the corresponding S/N. The concentration of (G) AR and (H) the corresponding S/N. The concentration of (I) G4 and (J) the corresponding S/N.

The optimal conditions were: 1.5 nM of TAC-dU2, 200 nM of hemin, 1.0 mM H_2O_2, 30 μM of AR and 0.4 μM of G4. These optimum conditions were adopted in further analysis.

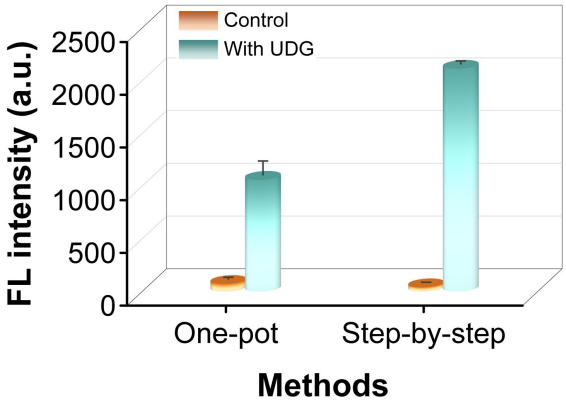

Figure S10. Comparison of the performances of the one-pot and step-by-step THRUST strategy.

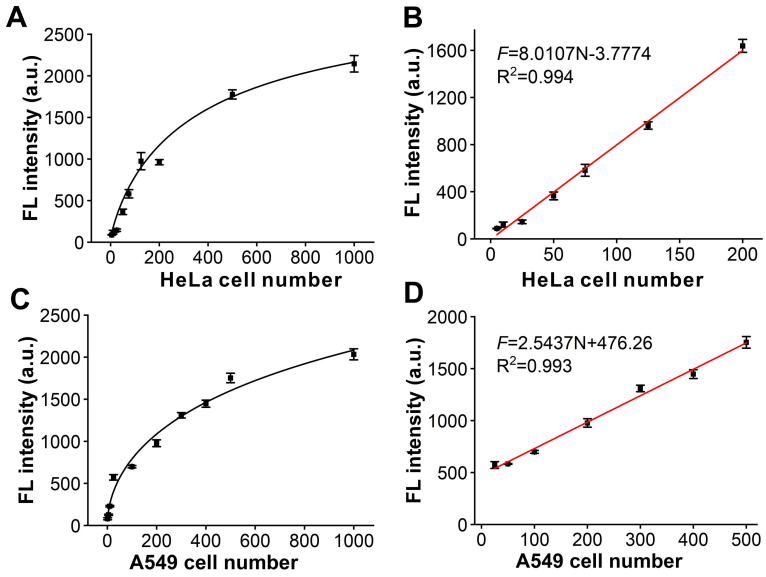

Figure S11. Real sample analysis towards cancer cells. (A) Fluorescence intensity of HeLa cell number from 5 to 1000 for THRUST system. (B) The linear relationship between absorbance intensity and HeLa cell number ranging from 5 to 200 cells. (C) Fluorescence intensity of A549 cell number from 5 to 1000 for THRUST system. (D) The linear relationship between absorbance intensity and A549 cell number ranging from 25 to 500 cells.

3. Sequences of oligonucleotides

Table S1. Sequences of oligonucleotides used in Figures 1-2 and S1.

Note	Sequence (5′-3′)
crRNA	UAA UUU CUA CUA AGU GUA GAU GAGA GAGA GAGA GAGA GAGA
T7 promoter	TAA TAC GAC TCA CTA TA GGG
Template	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
The complemenatry sequence of CRISPR array	TAA TTT CTA CTA AGT GTA GAT GAGA GAGA GAGA GAGA GAGA
TAC-dU2	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA T/ideoxyU/A CCC TA TAG TGA GTC GTA TTA
TAC-dU12	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT /ideoxyU/AG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-dU20	TCTC TCTC TCTC TCTC TCTC A/ideoxyU/C TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-dU27	TCTC TCTC TCTC TC/ideoxyU/C TCTC ATC TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-dU33	TCTC TCTC /ideoxyU/CTC TCTC TCTC ATC TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-dU39	TC/ideoxyU/C TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-AP2	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA T/idSp/A CCC TA TAG TGA GTC GTA TTA
TAC-AP12	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT /idSp/AG

	TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-AP20	TCTC TCTC TCTC TCTC TCTC A/idSp/C TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-AP27	TCTC TCTC TCTC TC/idSp/C TCTC ATC TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-AP33	TCTC TCTC /idSp/CTC TCTC TCTC ATC TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
TAC-AP39	TC/idSp/C TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA TTA CCC TA TAG TGA GTC GTA TTA
F-Q	FAM-TTT ATT-BHQ1

In the Template, the brown sequence represents the complementary sequence of T7 promoter, the blue sequence represents the T1 region in the CRISPR array, and the red sequence represents the T2 region in CRISPR array. ‘ideoxyU’ in the table represented the dU, ‘idSp’ in the table represented the AP site.

Table S2. Sequences of oligonucleotides used in Figures S3-S6.

Note	Sequence (5′-3′)
T7 promoter	TAA TAC GAC TCA CTA TA GGG
TAC-dU2	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA T/ideoxyU/A CCC TA TAG TGA GTC GTA TTA
TAC-AP2	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA T/idSp/A CCC TA TAG TGA GTC GTA TTA
F-Q	FAM-TTT ATT-BHQ1

‘ideoxyU’ in the table represented the dU. ‘ideoxyU’ in the table represented the dU, ‘idSp’ in the table represented the AP site.

Table S3. Sequences of oligonucleotides used in Figure 3, S7, S9 and S10.

Note	Sequence (5′-3′)
T7 promoter	TAA TAC GAC TCA CTA TA GGG
TAC-dU2	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA T/ideoxyU/A CCC TA TAG TGA GTC GTA TTA
TAC-AP2	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA T/idSp/A CCC TA TAG TGA GTC GTA TTA
G4	GGG TTA GGG TTA GGG TTA GGG

‘ideoxyU’ in the table represented the dU. ‘ideoxyU’ in the table represented the dU, ‘idSp’ in the table represented the AP site.

Table S4. Sequences of oligonucleotides used in Figure 4.

Note	Sequence (5′-3′)
T7 promoter	TAA TAC GAC TCA CTA TA GGG
TAC-dU2	TCTC TCTC TCTC TCTC TCTC ATC TAC ACT TAG TAG AAA T/ideoxyU/A CCC TA TAG TGA GTC GTA TTA
bio-G4	biotin-GGG TTA GGG TTA GGG TTA GGG

‘ideoxyU’ in the table represented the dU.

Table S5. Binding affinity assay of TAC and TAC-AP to crRNA.

DNA/RNA	ΔG (kcal/mol)
TAC/crRNA	-26.96
TAC-AP2/crRNA	-26.96
TAC-AP12/crRNA	-26.96
TAC-AP20/crRNA	-26.96
TAC-AP27/crRNA	-20.62
TAC-AP33/crRNA	-15.82
TAC-AP39/crRNA	-25.39

The data were calculated by the Integrated DNA Technologies (IDT) OligoAnalyzer (https://sg.idtdna.com/calc/analyzer).

Supplemenatry references

1. K. M. O'Hare and R. S. Hayward, *Nucleic Acids Res.*, 1981, **9**, 4689-4707.

2. S. T. Jeng, J. F. Gardner and R. I. Gumport, *J. Biol. Chem.*, 1992, **267**, 19306-19312.